

The Nramp (Slc11) proteins regulate development, resistance to pathogenic bacteria and iron homeostasis in *Dictyostelium discoideum*

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Summary

The *Dictyostelium discoideum* genome harbors two genes encoding members of the Nramp superfamily, which is conserved from bacteria (MntH proteins) to humans (Slc11 proteins). Nramps are proton-driven metal ion transporters with a preference for iron and manganese. Acquisition of these metal cations is vital for all cells, as they act as redox cofactors and regulate key cellular processes, such as DNA synthesis, electron transport, energy metabolism and oxidative stress. *Dictyostelium* Nramp1 (Slc11a1), like its mammalian ortholog, mediates resistance to infection by invasive bacteria. We have extended the analysis to the *nramp2* gene, by generating single and double *nramp1/nramp2* knockout mutants and cells expressing GFP fusion proteins. In contrast to Nramp1, which is recruited to phagosomes and macropinosomes, the Nramp2 protein is localized exclusively in the membrane of the contractile vacuole, a vesicular tubular network regulating cellular osmolarity. Both proteins colocalize with the V-H⁺-ATPase, which can provide the electrogenic force for vectorial transport. Like *nramp1*, *nramp2* gene disruption affects resistance to *Legionella pneumophila*. Disrupting both genes additionally leads to defects in development, with strong delay in cell aggregation, formation of large streams and multi-tipped aggregates. Single and double mutants display differential sensitivity to cell growth under conditions of iron overload or depletion. The data favor the hypothesis that Nramp1 and Nramp2, under control of the V-H⁺-ATPase, synergistically regulate iron homeostasis, with the contractile vacuole possibly acting as a store for metal cations.

Key words: *Dictyostelium discoideum*, Iron transporter, Nramp1, Nramp2, V-H⁺-ATPase, Iron homeostasis, Cell growth, Development, *Legionella pneumophila*, Bacterial infection, Phylogenesis

Introduction

The natural resistance-associated macrophage protein (Nramp) or solute carrier 11 (Slc11) family includes functionally related proteins defined by a conserved hydrophobic core of ten-twelve transmembrane domains. All members are proton-coupled transporters that facilitate absorption of divalent metal ions, including Fe²⁺, Mn²⁺, Zn²⁺, Co²⁺ and Cd²⁺ (Courville et al., 2006; Forbes and Gros, 2001; Nevo and Nelson, 2006;). In mammals two homologs are found, Nramp1 (Slc11a1) and Nramp2 (Slc11a2 or DMT1). Nramp1 is found exclusively in macrophages and granulocytes, is localized in vesicles of the endosomal pathway and recruited to the phagosomal membrane upon phagocytosis (Canonne-Hergaux and Gros, 2004; Gruenheid et al., 1997). By controlling divalent metal concentrations, Nramp1 regulates the intracellular replication of pathogens. In mice, naturally occurring or experimentally induced mutations in Nramp1 cause susceptibility to infections by invasive bacteria. Mutations in humans may genetically predispose an individual to diseases, including tuberculosis, leishmaniasis and autoimmune diseases (Courville et al., 2006; Li et al., 2011; Malik et al., 2005; Nevo and Nelson, 2006; Taylor and Kelly, 2010; Velez et al., 2009; Wick, 2011). In contrast to Nramp1, Nramp2 is localized in the plasma membrane of cells in several tissues, particularly the apical membrane of intestinal and renal epithelial cells, and is the major

transferrin-independent iron uptake system in mammals (Canonne-Hergaux et al., 1999; Courville et al., 2006; Theil, 2011). Alternative splicing gives rise to Nramp2 isoforms, which are mainly found in recycling endosomes, and are required in combination with Nramp1 for efficient iron recycling (Lam-Yuk-Tseung and Gros, 2006; Soe-Lin et al., 2010). Mutations in Nramp2 are responsible for microcytic anemia and serum and hepatic overload (Iolascon and De Falco, 2009). Genetic studies in rodents have shown that Nramp2 is essential for survival and a critical threshold of the protein is necessary to protect against disorders of iron and manganese homeostasis (Fleming et al., 1997; Gunshin et al., 2005).

Nramp proteins are widely distributed in eukaryotes and prokaryotes (Courville et al., 2006). Based on sequence analysis Richer et al. (Richer et al., 2003) have distinguished prototypical Nramps, present in amoebae, yeasts, plants and invertebrates, from archetypical Nramps, which can be also found in these species but are the only homologs present in vertebrates, including mammalian Nramp1 and 2. In Gram-positive and Gram-negative bacteria, the Nramp superfamily is represented by the proton-dependent manganese transporter (MntH) family (Papp-Wallace and Maguire, 2006; Richer et al., 2003).

The genome of the social amoeba *Dictyostelium discoideum* harbours two *nramp* genes. The first gene product that was

characterized was called Nramp1, as it displayed all the properties of the mammalian ortholog (Peracino et al., 2006). Similarly to mammalian Nramp1, *Dictyostelium* Nramp1 was expressed in vesicles of the endolysosomal pathway, was recruited to phagosomes, and its inactivation affected susceptibility to infection by *Legionella* and *Mycobacterium*. The protein was found to be required for H⁺-dependent iron export in isolated phagosomes (Peracino et al., 2006), favoring the notion of iron depletion from the phagosome, as originally suggested for mammalian Nramp1 (Forbes and Gros, 2001). Although Fe²⁺ in phagolysosomes can produce hydroxyl radicals via Fenton reaction, thus potentially killing bacteria, depletion of the iron pool from the phagosome deprives pathogenic bacteria of metal ions that are critical for their survival. Invasive bacteria, such as *Legionella pneumophila*, *Mycobacterium tuberculosis* or *Salmonella enterica* serovar *Typhimurium* are known to absorb large amounts of iron, while at the same time they are resistant to oxygen radicals (Henard and Vazquez-Torres, 2011; Johnson and Wessling-Resnick, 2012; Kortman et al., 2012; Moalem et al., 2004; Robey and Cianciotto, 2002; Schlauch, 2011). Recently, we also showed that *L. pneumophila* hinders recruitment of the V-H⁺-ATPase, but not Nramp1, in the *Legionella*-containing vacuole in a process apparently involving inhibition of phosphatidylinositol-3-phosphate formation. By avoiding colocalization of V-H⁺-ATPase with Nramp1 in the same vesicle, *Legionella* neutralizes the proton-driven iron export via Nramp1, and may even turn the protein activity to its advantage (Peracino et al., 2010).

Nramp1 is one of a few genes that have been implicated in *Dictyostelium* innate immunity against pathogenic bacteria (Bozzaro and Eichinger, 2011; Chen et al., 2007; Cosson and Soldati, 2008; Sillo et al., 2008; Steinert, 2011). *Dictyostelium* cells are professional phagocytes that live in the forest soil as solitary amoebae and grow by grazing on different species of bacteria (Bozzaro et al., 2008; Kessin, 2001). Depending on bacteria as the obligate source of food, *Dictyostelium* can also be a natural host of pathogenic bacteria, and several pathogens that only occasionally infect humans, including *L. pneumophila*, *M. tuberculosis*, *Salmonella typhimurium*, *Neisseria meningitidis* or *Pseudomonas aeruginosa*, have been shown to be pathogenic also for *Dictyostelium* (Bozzaro and Eichinger, 2011; Colucci et al., 2008; Hagedorn et al., 2009; Lima et al., 2011; Sillo et al., 2011; Steinert, 2011). The solitary *Dictyostelium* amoebae enter a social phase of life upon depletion of the food source. Starvation induces a developmental process, whereby cells assemble into multicellular aggregates by chemotaxis, giving rise to elongated slugs, where cells undergo differentiation into prestalk and prespore cells. After a period of migration, the slugs culminate and form fruiting bodies, each consisting of a stalk of vacuolated dead cells, bearing at the top a ball of mature spores (Kessin, 2001). During development, cells do not ingest external nutrients and are bound to recycle intracellular material, mostly by autophagy and protein degradation, for acquiring the energy and synthesizing all the cellular components required for development and cell differentiation (Kessin, 2001; Otto et al., 2003).

In this paper, we have characterized the second *nramp* gene of *Dictyostelium*, thus called *nramp2*. The *nramp2* gene is maximally expressed during the aggregation phase of development. We show that the protein is exclusively localized in the contractile vacuole network, a tubular-vesicular network

regulating osmolarity (Clarke et al., 2002; Clarke and Heuser, 1997; Heuser et al., 1993). By generating single and double *nramp1/nramp2* knockout mutants, we show that Nramp2, like Nramp1, is required for resistance to invasive bacteria, and that the double mutation leads additionally to a strong delay in aggregation and to formation of multi-tipped aggregates. Under conditions of iron overload or depletion, growth of single or double KO mutants is differentially affected, suggesting that the Nramp proteins regulate synergistically iron homeostasis.

Results

Characterization and developmental expression of the *nramp2* gene

The *Dictyostelium* genome contains two genes, both located in chromosome 2, with evident sequence homology to genes of the Nramp superfamily. The *nramp1* gene (DDB_G0276973) is the ortholog of mammalian *nramp1* and was characterized previously (Peracino et al., 2006; Peracino et al., 2010). The *nramp2* gene (DDB_G0275815) encodes a putative integral membrane protein with 12 transmembrane domains and a *M_r* of 70 kDa, 10 kDa larger than Nramp1 (Fig. 1). The DNA sequence is intronless, in contrast to *nramp1*, which harbours an intron at position 199–309. The protein sequence shares 38% identity and 75% similarity with Nramp1, and the similarity, as expected, is low in the N- and C-terminal outer membrane sequences (Fig. 1). Blast analysis against reference proteins placed Nramp2 within the Nramp superfamily (Fig. 2). It is phylogenetically closer to α -proteobacteria MntH and to Nramp proteins from yeast, fungi and protists than to the Slc11 family, to which instead Nramp1 belongs (Fig. 2) (Richer et al., 2003). We extended this analysis to include all Nramp1 and Nramp2 homologs encoded in sequenced genomes of *Dictyosteliaceae*. As shown in Fig. 2, Nramp1 and Nramp2 homologs from *D. discoideum*, *D. purpureum*, *D. fasciculatum*, *D. lacteum* and *Polysphondylium pallidum* cluster in two relatively distinct groups. This is in sharp contrast with other lower eukaryotes, such as *Chlamydomonas reinhardtii*, and *Saccharomyces cerevisiae*, which harbor respectively 2 and 3 Nramps, forming in both cases, however, a single group (Fig. 2). *Arabidopsis thaliana* possesses 6 Nramps clustering in two groups, both of which are more closely related to *Dictyostelium* Nramp1 than Nramp2.

Whereas *nramp1* gene expression is higher during growth and declines upon starvation remaining at low level during development (Fig. 3) (Peracino et al., 2006), *nramp2* gene expression was very low during growth, increased during the first 6 hours of development and declined after formation of tight aggregates (Fig. 3). This pattern is similar to that revealed by RNA-seq analysis, available from the *Dictyostelium* webpage (www.dictybase.org).

Nramp2 is a resident membrane protein of the contractile vacuole

To determine the cellular localization of Nramp2 we cloned the gene and generated a chimeric protein with green fluorescence protein (GFP) fused at the C-terminus. Nramp2(C)-GFP homogeneously labeled the membrane of the contractile vacuole (CV) network (Fig. 4A). In living cells plated on glass, the CV network, made of branched vesicles and tubules, is typically located near the ventral surface of the cell, and extends over a large area close to the plasma membrane, as can be better appreciated in a 3D reconstruction (supplementary material

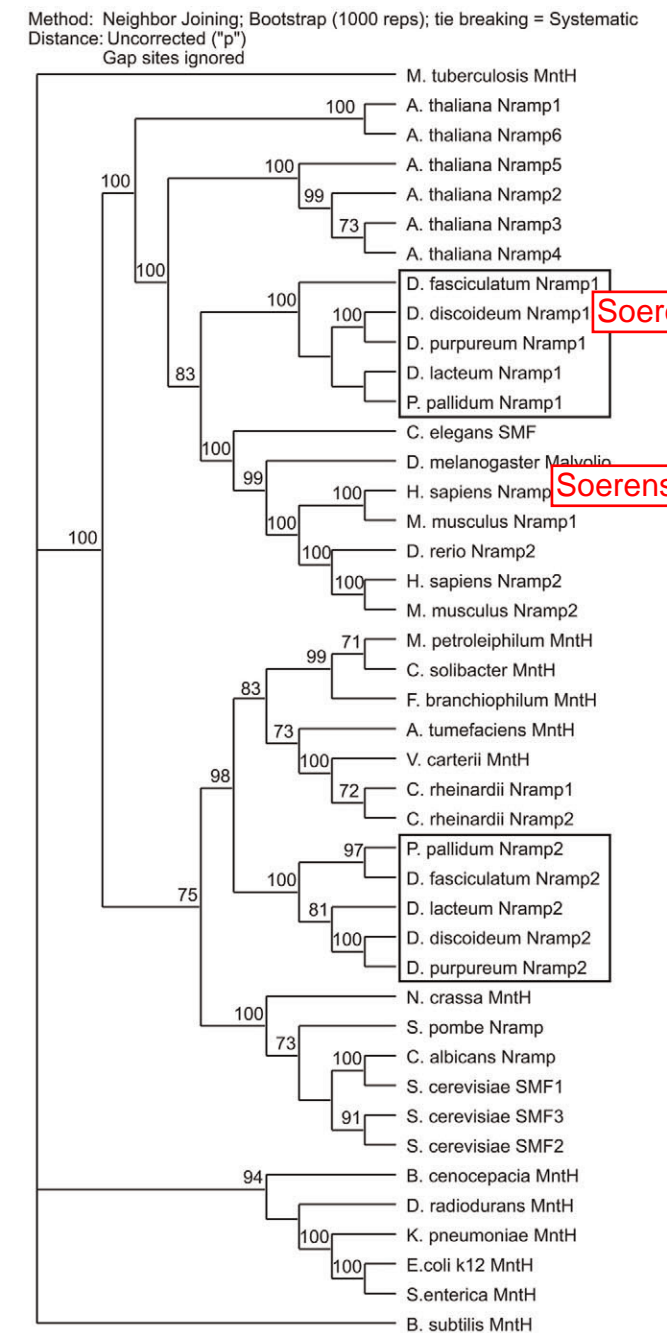


Fig. 2. Phylogenetic relationship of *Dictyostelium discoideum* Nramp1 and Nramp2. *Dictyostelium discoideum* Nramp1 and Nramp2 sequences were compared to similar sequences using the MacVector software and performing multiple sequence alignment with the program ClustalW program (Blosom matrix). The evolutionary distance was inferred using the neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Only a few selected sequences close to each protein are shown. *Dictyostelium* Nramp1 and Nramp2, together with homologs from the other *Dictyosteliaceae*, whose genomes have been sequenced, cluster in two different groups, the first closer to Nramp proteins of the Slc11a family and the second to yeast SMF and α -proteobacteria MntH family.

double mutant formed larger aggregating streams on phosphate agar, which led to formation of large multitipped aggregates (Fig. 5A,B). Despite aggregate fragmentation due to multiple tip

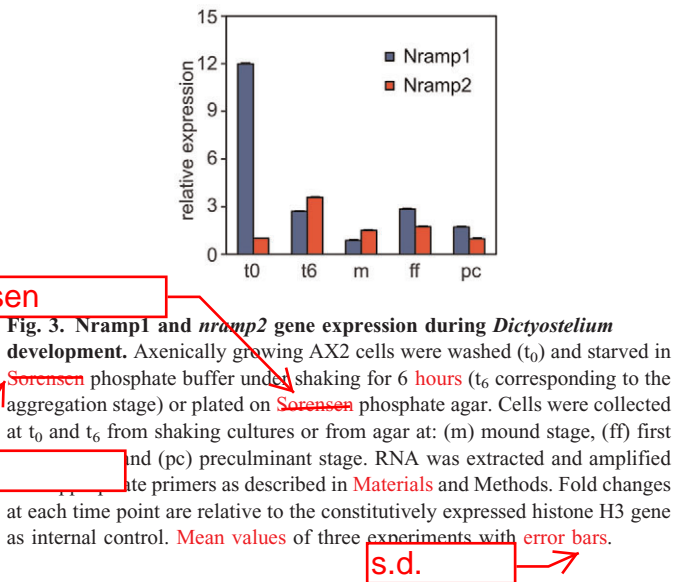


Fig. 3. Nramp1 and *nramp2* gene expression during *Dictyostelium* development. Axenically growing AX2 cells were washed (t_0) and starved in phosphate buffer under shaking for 6 hours (t_6 corresponding to the aggregation stage) or plated on phosphate agar. Cells were collected at t_0 and t_6 from shaking cultures or from agar at: (m) mound stage, (ff) first and (pc) preculminant stage. RNA was extracted and amplified using the primers as described in Materials and Methods. Fold changes at each time point are relative to the constitutively expressed histone H3 gene as internal control. Mean values of three experiments with error bars. s.d.

formation, the density of fruiting bodies was markedly reduced compared to the parental strain, and the fruiting bodies were on the average taller, though the proportion between sori and stalks was maintained (Fig. 5C,D). Expressing either Nramp1 or Nramp2-GFP in the double mutant led to rescue of both developmental timing and aggregate morphology compared to the single mutants expressing either Nramp1- or Nramp2-GFP, respectively.

Differential effects of iron depletion or overload on mutant growth in minimal medium

The Nramp proteins are proton-driven divalent metal transporters, with a preference for iron and manganese. We have shown this to apply also for *Dictyostelium* Nramp1 (Peracino et al., 2006), and we assume that Nramp2 also displays this function, based on the conserved protein sequence and general protein structure. As a first step to assess potential effects of *nramp1* or *nramp2* gene disruption on iron homeostasis, we tested the effects of iron on cell growth, by assaying growth in a minimal FM medium, containing aminoacids, vitamins, glucose and essential trace elements, previously shown to support *Dictyostelium* cell growth (Franke and Kessin, 1977). The FM medium contained 0.1 mM FeCl_3 . We used the same recipe, but either omitted iron or added 0.01, 0.1 or 0.2 mM FeCl_3 . AX2 or mutant cells exponentially growing in AX2 medium were diluted 100-fold in minimal medium to an initial concentration of $3\text{--}5 \times 10^4$ cells per ml, and growth was assessed under shaking over a period of 2 to 4 weeks. Despite variability in the absolute values from experiment to experiment, the trend was similar. In culture medium without added iron, only the parental AX2 cells displayed steady though slow growth, with a doubling time of about 35–40 hours, reaching a density of 1×10^6 cells per ml after 168 ± 24 hours (Fig. 6). The AX2 cells underwent on the average five to six duplications before reaching a plateau. The single as well as the double *nramp1/nramp2* KO mutants grew only up to $1\text{--}2 \times 10^5$ cells per ml during the first 72 to 96 hours incubation (doubling time: 36 to 45 hours), but then stopped growing (Fig. 6). Minimal medium supplemented with 0.01 (not shown) or 0.1 mM iron (Fig. 6) supported growth of AX2, single and double-null mutants, with all strains reaching a

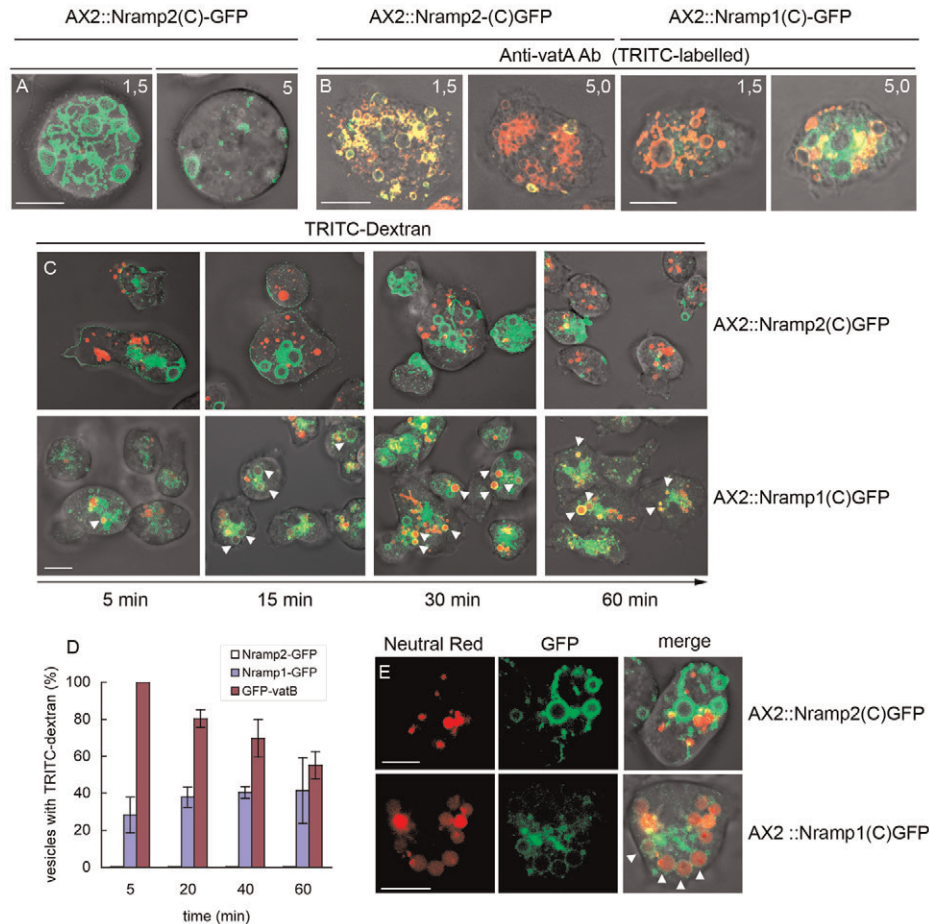


Fig. 4. Dynamics of Nrapm2 and Nrapm1 in cells visualized with GFP fusion protein. (A) Confocal GFP fluorescence sections merged with corresponding phase contrast through living AX2 cells expressing Nrapm2(C)-GFP. The numbers indicate distance from the bottom surface of the cell. Nrapm2(C)-GFP labels homogeneously vesicles interconnected by tubules and localized at the bottom region of the cell close to the substratum, which is typical of the contractile vacuole. In upper regions no labeling independent of the contractile vacuole is observed. (B) Confocal GFP fluorescence sections merged with corresponding phase contrast of methanol-fixed AX2 cells expressing either Nrapm2(C)- or Nrapm1(C)-GFP and costained with antibodies against the VatA subunit of the V-H⁺-ATPase. The Nrapm2-decorated vesicles in the bottom region colocalize fully with the VatA-labeling, confirming that this structure is the contractile vacuole. The V-H⁺-ATPase subunit is also found in endolysosomal vesicles in the upper region that are not labeled by Nrapm2(C)-GFP, indicating that Nrapm2 is not expressed in endolysosomes. Nrapm1 is not found in the contractile vacuole but in a cluster around the nucleus (Golgi) and in some vesicles colabeled with anti-VatA antibodies. Notice that methanol fixation affects in part Nrapm1 and Nrapm2-GFP fluorescence. Bars: 5 μ m. (C) Confocal GFP fluorescence merged with corresponding phase contrast of living AX2 cells expressing either Nrapm2(C)- or Nrapm1(C)-GFP. The cells were incubated for 5 minutes with TRITC-dextran, washed and plated on glass coverslips for the indicated chase time. In contrast to Nrapm1-GFP, which decorates TRITC-dextran containing vesicles (arrowheads), Nrapm2-GFP fails to colocalize with TRITC-dextran, even in cells with a thin fluorescent rim of Nrapm2-GFP on the plasma membrane. (D) The number of TRITC-dextran positive vesicles decorated with GFP-fused Nrapm1, Nrapm2 or the vatB subunit of the vacuolar ATPase was plotted over time. For each time point an average of 50 macropinosomes and 15 cells were counted. Mean values of three independent experiments with error bars are shown (data for the VatB subunit and Nrapm1 are reported from Peracino et al. (Peracino et al., 2006)). (E) Confocal sections of cells expressing Nrapm1(C)- or Nrapm2(C)-GFP incubated with neutral red, a marker of acidic vesicles. Red, green fluorescence and merge with corresponding phase-contrast are shown sequentially. Nrapm2 fails to colocalize with acidic vesicles, in contrast to Nrapm1, which decorates some vesicles (arrowheads). Bars: 5 μ m.

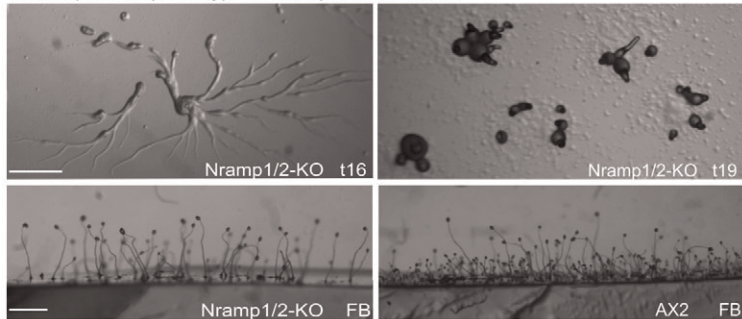
concentration of 1×10^7 cells per ml after 168 to 196 hours incubation. At a concentration of 0.2 mM iron, growth was inhibited in all strains, more strongly in the parental strain, but remarkably less so in the double null mutant, which was the only one able to steadily grow to 3×10^6 cells per ml after 168 hours of incubation, with an average doubling time of about 26 hours (Fig. 6). After two doublings in the first 48 hours, the Nrapm2 KO mutant strain took a further 120 hours to undergo a third doubling. The Nrapm1 mutant and the parental AX2 strain doubled once in the first 24 hours and then needed about 100 and

140 hours, respectively, to double a second time. Thus it appears that 0.2 mM iron is somewhat toxic, particularly for wild type cells, whereas the double null mutant is relatively resistant. In contrast, all three mutants are much more sensitive to iron shortage than the parental strain. Cells transferred from axenic to the minimal medium contain iron released from digestion of peptone and yeast extract, and traces of iron are possibly available in the minimal medium even without added iron, thus it is not surprising that cells may undergo some duplication rounds. The results suggest nevertheless that inactivating either *nrapm1*

Effects of single or double gene disruption on developmental timing

Strain	aggregation	tip mounds	first fingers	pre-culminants	fruiting bodies
AX2	6	12	16	20	24
Nramp1-null	6	9	12	17	20
Nramp2-null	8	14	16	20	24
Nramp1/2-null	12	18	20	23	27

Developmental phenotype of Nramp1/2 null mutant



Soerensen
Fig. 5. Effects of single or double gene disruption on development. Starving cells of parental strain and mutants were plated on non-nutrient Sørensen-phosphate agar and development was monitored over time in a stereomicroscope. **Top panel:** the time (in hours) needed for beginning of stream formation (aggregation) or appearance of tipped aggregates, first fingers, pre-culminants and mature fruiting bodies is indicated for each mutant. (Mean of two experiments. The standard deviation was of ± 1 hour or 2 hours for aggregation or the other developmental stages, respectively.) **Bottom panels:** developmental phenotype of the double mutant. (A) Cells aggregating after 12 hours starvation formed very large streams and (B) multi-tipped aggregates. (C) On the average taller fruiting bodies, but of reduced number, were formed, compared with (D) AX2 parental strain. Bars: 1 mm.

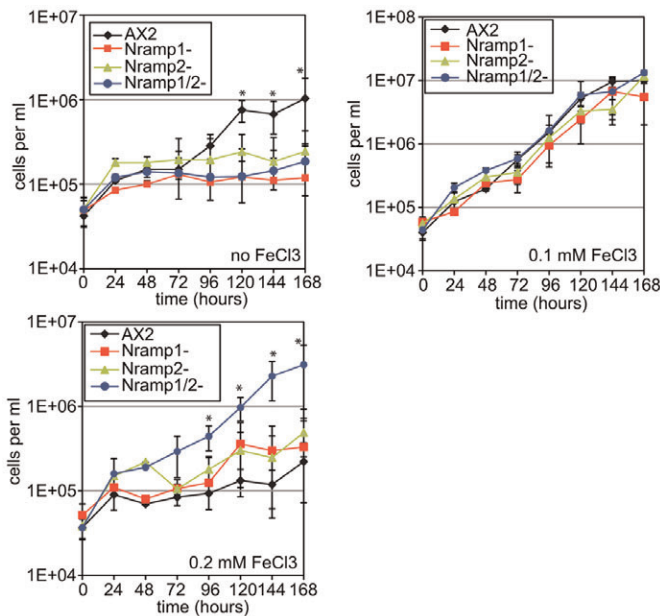


Fig. 6. Effects of single or double gene disruption on growth in minimal medium with or without FeCl₃ supplementation. Exponentially growing cells were diluted in minimal medium with or without the indicated amount of iron chloride, incubated under shaking and counted for growth at the indicated times. In the absence of iron, cell growth was blocked after 1 or 2 generations in all mutants, whereas the parental AX2 strain was able to replicate for 4–5 generations, albeit very slowly. All strains grew well with 0.1 mM iron, whereas with 0.2 mM iron growth was depressed in all strains, but much less so in the double mutant. Mean values of at least three experiments with s.e.m. are shown. The asterisks denote significant difference (one-tailed *t*-test assuming unequal variance; *P* < 0.05) between AX2 versus the double mutant in the first graph (no iron) or the double mutant versus AX2 in the third graph (0.2 mM iron).

or *nramp2* renders the cells more sensitive than wild type cells to conditions of iron depletion. In contrast, inactivating both genes results in increased resistance to iron overload.

Nramp2, like Nramp1, is required for resistance to *L. pneumophila* infection

We showed previously that Nramp1 KO mutants were more susceptible to infection by *L. pneumophila* and *Mycobacterium avium* (Peracino et al., 2006; Peracino et al., 2010). This prompted us to test whether disrupting Nramp2 also affected resistance to invasive bacteria and whether the double mutation resulted in any additional effect. Cells were incubated with *L. pneumophila* and the rate of bacterial growth was assessed by the CFU (Colony Forming Unit) method. Despite high variation in the absolute values from experiment to experiment, the trend was similar in four different tests, as shown in the representative experiment in Fig. 7. The bacteria proliferated as well in the single *nramp2*- as in the *nramp1*-null mutant, and significantly better than in the parental AX2 strain. The double mutant behaved similarly to the single mutants up to 72 hours of infection, thereafter bacterial proliferation consistently declined. Visual examination showed, however, that at 96 hours there were clearly more extracellular bacteria in the medium containing cells of the double mutant than the single mutants, yet this finding escaped CFU detection. Since CFU measures viable bacteria, and the culture medium used for the infection assay does not support bacterial proliferation (Fig. 7) (Hägele et al., 2000; Peracino et al., 2006), these results are consistent with the hypothesis that between 72 and 96 hours of infection a higher number of cells undergo lysis in the double knockout than in the single mutants, releasing more bacteria into the medium. Because the released bacteria die in the assay medium, their higher number is not reflected in the subsequent CFU counts. Attempts to measure bacterial proliferation using GFP-labeled bacteria in fluorescence-activated flow cytometry failed, as GFP expression diminished drastically after 48 h of incubation.

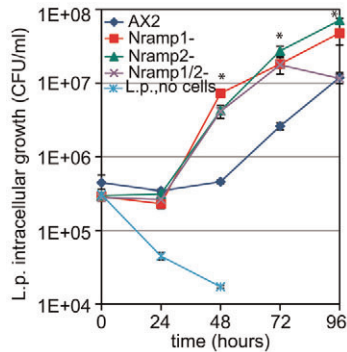


Fig. 7. Effects of single or double gene disruption on infection by *Legionella pneumophila*. AX2 parental strain and mutants were infected with *L. pneumophila* (L.p.) and intracellular growth of the bacteria was assessed by the CFU assay at the time indicated in the abscissa. In medium without cells, the bacteria do not survive. Mean values of a representative experiment in duplicate with error bars are shown. The asterisks denote significant differences compared to the parental strain for all three mutants at times 48 and 72 hours, and at time 96 hours for the Nrapm1- and Nrapm2-null mutant only (one-tailed, assuming unequal variance: $P < 0.05$). The trend in four different experiments was similar, although the absolute values varied strongly from experiment to experiment.

Discussion

The strong conservation in sequence and overall transmembrane structure in the Nrapm family reflects the fact that proton-driven metal cation transport is conserved from prokaryotes to higher eukaryotes (Courville et al., 2006; Czachorowski et al., 2009). Iron is vital for all cells, as it modulates the cellular redox potential and regulates key cellular processes, from electron and oxygen transport to energy metabolism and DNA synthesis (Hentze et al., 2010; Kakhlon and Cabantchick, 2002; Schaible and Kaufmann, 2004). Despite the phylogenetic conservation of the Nrapm proteins, *D. discoideum* is unusual in that it contains to distant Nrapm proteins, Nrapm1 in the Slc11 family, and Nrapm2 which is more closely related to yeast SMF, *C. reinhardtii* Nrapm and to α -proteobacteria MntH proteins. We have shown this to be true for all five Dictyostelids whose genomes have been sequenced. This is in sharp contrast with other lower eukaryotes harbouring more than one Nrapm paralogs, such as *C. reinhardtii*, *S. cerevisiae* or *A. thaliana*, whose Nrapm proteins form in each case a single cluster or, in the case of *A. thaliana*, two different, but very closely related clusters. It has been suggested that Nrapm proteins originated in prokaryotes and were transferred endosymbiotically to the eukaryotic nucleus. Horizontal gene transfer from eukaryotes to prokaryotes has also been hypothesized to justify unexpected sequence diversity in MntH phyletic groups (Richer et al., 2003), and could explain the surprisingly closer similarity of Dictyostelid Nrapm2 to α -proteobacterial homologs than to other bacterial MntH sequences. If the Slc11 Nrapm family derived from a duplication from an ancient Nrapm, as is likely (Richer et al., 2003), then *Dictyostelium* offers a unique opportunity to study the evolutionary divergence of these two types of Nrapm proteins coexisting in a single organism.

The cellular localization of the Nrapm1 and Nrapm2 is very different. Like mammalian Nrapm1, *Dictyostelium* Nrapm1 shuttles from the Golgi to the endolysosomal pathway, being recruited to phagosomes and macropinosomes shortly after their

closure and recruitment of the V-H⁺-ATPase. Colocalization of V-H⁺-ATPase and Nrapm1 in the membrane generates the right conditions for proton-driven transport of iron and other metals from such vesicles to the cytosol, exactly as occurs for vertebrate Nrapm1 (Courville et al., 2006; Peracino et al., 2006). Nrapm2 is instead expressed selectively in the membrane of the contractile vacuole network, which regulates osmolarity. As soil amoebae living in the forest *Dictyostelium* cells are exposed to sudden changes of osmotic conditions, so that water regulation is critical for their survival. The contractile bladder and tubular network fills with water and swells under hypoosmotic conditions, giving rise to larger vacuoles that fuse with the plasma membrane to discharge excess fluid. Under hyperosmotic conditions, the cisternae flatten and the tubules get narrow (Clarke and Heuser, 1997; Gerisch et al., 2002; Heuser et al., 1993). With a functioning CV, cells submerged in water survive, acquire the typical amoeboid shape and can also undergo development, whereas they rapidly round up and lyse if the CV cannot fulfil its function (Du et al., 2008; Gerald et al., 2002; Myre et al., 2011). The CV membrane is studded with vacuolar H⁺-ATPase, which pumps protons into the lumen. Cells possess also a cytoplasmic carbonic anhydrase and a bicarbonate transporter in the CV membrane (Gigliome and Gross, 1995; Heuser et al., 1993). It has been suggested that production of bicarbonate from CO₂ by the carbonic anhydrase and cotransport of the anion with protons into the lumen of the CV generate osmotically active carbonic acid that could draw water into the vacuole (Clarke and Heuser, 1997; Heuser et al., 1993).

The finding that V-H⁺-ATPase and Nrapm2 co-localize in the CV membrane is suggestive of a potential role of the contractile vacuole in homeostasis of iron, and possibly other divalent metals. In the wild the major source of iron for *Dictyostelium* cells is very likely the engulfed bacteria. We propose that iron, released after phagocytosis and digestion of bacteria (or ingested with peptone and yeast extract by macropinocytosis, in the case of laboratory axenic strains) is reduced to Fe²⁺ in phago- or macropinosomes and transported via Nrapm1 to the cytosol, where it can be used for cellular functions. Excess free iron could be transported in the CV that could thus function as a store or sink for the labile iron pool. The colocalization of Nrapm1 or Nrapm2 with the V-H⁺-ATPase in phagolysosomes or the contractile vacuole, respectively, could explain vectorial transport by a common mechanism, namely electrogenic potential driven by the proton pump (Fig. 8). It is established for mammalian Nrapm1 and Nrapm2 (Courville et al., 2006), *Dictyostelium* Nrapm1 (Peracino et al., 2006), and for bacterial and yeast homologs of *Dictyostelium* Nrapm2 (Courville et al., 2006), that the proton gradient generated by the vacuolar ATPase favors iron export from vesicles, such as phagolysosomes or yeast vacuoles, via Nrapm. In the case of *Dictyostelium* Nrapm2, this mechanism of action would imply iron efflux from the contractile vacuole into the cytosol (Fig. 8). One objection to this mechanism is that the pH in the CV lumen is neutral, possibly due to proton buffering by cotransported bicarbonate (Clarke and Heuser, 1997; Gigliome and Gross, 1995). It is possible however that a slight Δ pH across the CV membrane is present, which could be sufficient for iron efflux via Nrapm2. In this context it is worth mentioning that a P-type Ca²⁺ ATPase (PAT1) is also localized to the CV and is involved in V-H⁺-ATPase-dependent Ca²⁺ sequestration (Moniakakis et al., 1999), as well as efflux to the cytoplasm, although it is unclear how the latter is regulated

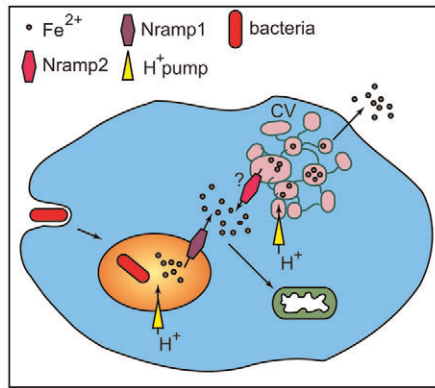


Fig. 8. Model for the function of Nramp1 and Nramp2 and of the contractile vacuole (CV) in iron homeostasis. Nramp1 is recruited to phagosomes after bacterial uptake and recruitment of the V-H⁺-ATPase to the phagosomes, whereas Nramp2 and the V-H⁺-ATPase are resident proteins of the CV network. Acidification by the V-H⁺-ATPase leads to a proton gradient in the phagosome that favors cotransport to the cytosol of ferrous ions deriving from bacterial degradation (or digestion of peptone and yeast extract in case of growth in axenic medium). Iron is required for cellular functions, particularly in mitochondria. We suggest that the contractile vacuole act as a store/sink for labile iron, with sporadic or induced CV fusion with the plasma membrane leading to iron excretion. Iron can be transported across the CV membrane via Nramp2, and the directionality of this transport is regulated by the V-H⁺-ATPase, which can provide the electrogenic potential across the membrane for iron influx into the cytosol, **similar** to what occurs in the phagosome. It is worth mentioning however that the pH in the CV lumen is mostly neutral (Gigliione and Gross, 1995), therefore it is open **to what extent** this mechanism is constitutively active or efficient.

(Malchow et al., 2006). In any case a role for Nramp2 in mobilizing iron from the contractile vacuole is suggested by the iron-limited growth as well as the developmental delay of the *nramp2*-null mutant. If the model in Fig. 8 is correct, it can be speculated that *Dictyostelium* Nramp2 plays a role similar to the Nramp homologs in yeast (SMF3) or *A. thaliana* (AtNramp3 and AtNramp4), which mobilize iron from their respective vacuoles (Portnoy et al., 2000; Lanquar et al., 2005).

We have shown that disrupting *nramp1*, *nramp2* or both has pleiotropic effects on development, cell growth under iron overload or shortage and on resistance to *L. pneumophila*. The developmental phenotypes are more difficult to correlate with specific defects in iron homeostasis, as very little is known in this regard in *Dictyostelium*. Development occurs in a simple salt solution made of Na- and K-phosphate, and can also occur in water. Exogenous iron or other metals are not essential, thus iron that is certainly required for mitochondrial activity and other iron-linked enzymatic activities is all fuelled by endogenous store. It is of interest that Nramp1 gene expression is downregulated during development, whereas Nramp2 expression is upregulated during the first 6 hours of development, coincident with the aggregation stage, and downregulated after formation of tight aggregates. Consistent with this regulation, Nramp1 gene disruption has little effect on development, resulting only in a slight acceleration of development, whereas inactivating Nramp2 delays the beginning of aggregation by 2 hours. Inactivating both genes results in a much stronger delay in cell aggregation, in large streaming aggregates and multitipped mounds. Despite

fragmentation following multitip formation, fewer but on average taller fruiting bodies are formed in the double mutant compared to the parental strain. This phenotype can be explained by assuming that development of the aggregation competence is inhibited in the double mutant, such that fewer cells in the population are able to initiate autonomous cell-cell signaling that is required for chemotaxis and aggregation. It can be speculated that altered iron homeostasis affects mitochondrial activity which is necessary for proceeding in development. It is worth mentioning that starvation induces upregulation of mitochondrial enzymes, such as the citrate synthase. In addition, many Krebs cycle as well as oxidative phosphorylation enzymes and the iron-sensing aconitate hydratase are strongly upregulated during stream formation and aggregation up to the mound stage (Czarna et al., 2010). Thus, reduced mitochondrial efficiency, as a consequence of defective iron storage during growth and/or mobilization during development, could explain the delay of the single Nramp2 mutant and the developmental phenotype of the double mutant. It is suggestive that large streams and multitipped aggregates are also a common phenotype of autophagy mutants (Otto et al., 2003; Tung et al., 2010). Defective autophagy could also result in altered oxidative phosphorylation, as autophagy is required for recycling of intracellular components during *Dictyostelium* development (Otto et al., 2003).

The major effect of *nramp1* inactivation is increased susceptibility to infection by *L. pneumophila* and *M. avium* (Peracino et al., 2006), resembling in this regard the effects of mutated mammalian Nramp1 (Cellier et al., 2007). Inactivating *nramp2* or both genes also leads to increased sensitivity to *Legionella* infection compared to the parental strain, with the double mutant being very likely more affected, although this was not reflected in increased bacterial proliferation when measured by the CFU method. As mentioned in the results, this may be due to the CFU assay measuring bacteria that were viable at the time of the assay and not bacteria that had been released prior to the assay but subsequently died in the inhospitable culture medium. Thus it appears that any alteration of iron homeostasis via Nramp1 or Nramp2 disruption facilitates intracellular growth of *Legionella*. Whether this holds true also for *M. avium* is currently under study. In the absence of Nramp1, iron export from phagosomes or macropinosomes is strongly reduced (Peracino et al., 2006), thus it is conceivable that disruption of the *nramp1* gene will lead to increased iron accumulation in the *Legionella*-containing macropinosome, a condition favoring *Legionella* growth (Robey and Cianciotto, 2002). How can inactivation of Nramp2, which is localized in the contractile vacuole, have a similar effect, if Nramp1 is active? We have recently shown that *Legionella* effectively hinders recruitment of the V-H⁺-ATPase, but not Nramp1, to the *Legionella*-containing macropinosome (Peracino et al., 2010). In the absence of the vacuolar ATPase, Nramp1 pumping activity is neutralized, and iron may be sequestered by the *Legionella* (Peracino et al., 2006). Under these conditions, if Nramp2 is not functioning it is possible that cytosolic iron deficiency may affect basic cell functions, favoring *Legionella* growth.

In contrast to pathogens, non-pathogenic bacteria, such as *E. coli*, are taken up and digested at about the same rate by the mutants as well as the parental strain. Cell growth in either axenic medium, presumably containing about 0.05 mM Fe (Franke and Kessin, 1977), or minimal medium supplemented with 0.01 to

0.1 mM iron chloride, is also not dramatically affected by *nramp1* or *nramp2* gene inactivation. In contrast, the single and double mutants are much more sensitive than the wild type strain to iron depletion and less sensitive, particularly the double mutant, to iron overload. Both results are consistent with the assumption that inactivating Nramp1, Nramp2 or both leads to lower accumulation of cytosolic iron, decreasing iron availability for cellular functions. As a consequence, when shifted from axenic medium to conditions of iron shortage the mutant cells deplete their free iron reserves more rapidly and stop growing after 1–2 duplications, whereas the parental wild type strain is able to sustain about 5–6 duplication rounds. In the case of iron overload, growth is strongly reduced in all strains, but the double mutant is more resistant to excess iron than the wild type strain and is able to grow, albeit slowly. Indeed, under the experimental conditions of this study most iron is very likely taken up via macropinocytosis, a process that is not affected by disruption of *nramp1* or *nramp2*. However, Nramp1 loss is expected to strongly reduce iron release from the macropinosomes to the cytosol, as iron transport across the phagosomal membrane was found to be inhibited in *nramp1*-KO mutant (Peracino et al., 2006). In contrast to many mammalian cells, in *Dictyostelium* the endolysosomes are not the dead-end of endosomal traffic. Instead they mature into neutral post-lysosomal vesicles that fuse with the plasma membrane, releasing their contents. Thus, if Nramp1 is inactive, iron will remain in large part entrapped in macropinosomes and could be excreted. This can explain the phenotype of both the single Nramp1 and the double Nramp1/Nramp2 KO mutant. The higher resistance of the latter to iron overload, compared to the parental AX2 strain and to the single mutants, could depend both on less iron leaking from macropinosomes and from the contractile vacuole, due to absence of Nramp1 and Nramp2. However, disruption of both genes may also affect expression of other potential regulators of iron homeostasis, such as ferritin-like proteins, which appear to be encoded in the *Dictyostelium* genome. Cloning and analysis of these genes will be required for a better understanding of iron homeostasis in *Dictyostelium*.

In summary, the described phenotypes can be explained by the model presented in Fig. 8, according to which Nramp1 and Nramp2, due to their conserved function, distinct intracellular location and to colocalization with the vacuolar ATPase, synergistically regulate proton-driven iron transport across the phagosomal/macropinosomal membrane and the membrane of the contractile vacuole.

Material and Methods

Cell and bacterial strains and culture methods

Dictyostelium discoideum parental strain AX2 was used. The Nramp1-null mutant HSB60 and AX2 cells expressing Nramp1-GFP had been generated previously (Peracino et al., 2006).

All strains were cultured axenically in either AX2 medium (Watts and Ashworth, 1970) or minimal medium (Franke and Kessin, 1977), with or without FeCl₃. In both cases, cells were grown in Erlenmeyer flasks at 23°C under shaking at 150 r.p.m. on a gyratory shaker in a climatic cabinet (Kuehner, Basel, Switzerland) as described (Bussolino et al., 1991). Blastidicin at a concentration of 10 µg/ml was added to knockout mutants. Cells expressing GFP-fused proteins were cultured in the presence of 10 or 20 µg/ml G418.

For growth and development on nutrient agar, cells were mixed with *Escherichia coli* B/2, plated on nutrient agar plates containing peptone and glucose in Soerensen phosphate buffer and incubated at 23°C (Sillo et al., 2008). Growth and development were observed in a Wild M3Z stereomicroscope and pictures were taken with a Bresser PC-Microcual 3.0 MP webcam connected to a PC-computer, using Webcam Companion 1 software.

Legionella pneumophila Corby expressing GFP was grown on buffered charcoal yeast extract agar (BYCE) containing 5 µg/ml chloramphenicol and incubated for 72 hours at 37°C and 5% CO₂ (Fajardo et al., 2004).

Cell development on non-nutrient agar plates

For synchronizing development, exponentially growing cells were harvested from culture medium, washed twice in 0.017 M Soerensen Na/K phosphate buffer, pH 6.0 and resuspended in the same buffer at a concentration of 1×10^7 per ml. Aliquots of 0.05 ml were plated on agar plates buffered with Soerensen phosphate buffer at a density of about 3×10^5 cells per cm², and incubated at 23°C (Bozzaro et al., 1987).

Infection assays with *L. pneumophila*

Exponentially growing AX2 and mutant cells were washed, incubated in AX2 medium without maltose and aliquots of 1×10^5 cells were plated in 96-well tissue culture plates. Freshly collected *L. pneumophila* were added at a MOI of 1:1 and immediately centrifuged at 600 g for 10 min at room temperature to synchronize infection. The number of viable bacteria was calculated by counting the colony forming units (CFU) per ml immediately after centrifugation and during the subsequent 96 hours (Bozzaro et al., 2012).

Gene cloning, vector construction for GFP expression and cell transformation

The *nramp2* gene (DDB_G0275815) was cloned starting from the genomic DNA fragment JC2a11a12 amplified with appropriate primers and cloned in pGEM-TEasy (Promega, Madison, WI, USA), according to Sambrook et al. (Sambrook et al., 1989). For fusion with GFP, the Nramp2-coding sequence was amplified from pGEM-TEasy-Nramp2 and inserted into the *EcoRI* site of the pDEX-GFPC vector with Nramp2(C) connected to S65T-GFP by the linker EFKKLLK (Westphal et al., 1997). Cell transformation was done by electroporation (Pang et al., 1999) and transformants were selected on plates with nutrient medium containing 10 µg/ml G418.

Construction of single *nramp2* and double *nramp1/2* knockout mutants

The blastidicin cassette was excised from the pLPBLP(floxed Bsr) vector (Faix et al., 2004) in *SmaI*, purified and inserted in the single *EcoRV* site of the Nramp gene in pGEM-TEasy-Nramp2. The vector carrying the selectable marker was linearized from the pGEM-TEasy vector with *EcoRI*, purified and electroporated in AX2 cells. The transformed cell population was splitted in 96-well plates and grown in the presence of 10 µg/ml of blastidicin. Resistant clones were screened by PCR using appropriate primers. Positive clones were subject to Southern blot analysis as described (Bracco et al., 1997). After a preliminary phenotypic analysis of three clones, one clone was treated with pDEX-NLS-cre to transiently express the CRE recombinase and remove the blastidicin cassette (Kimmel and Faix, 2006). To generate the double mutant, one clone was further transformed with the linearized Nramp1 gene containing the bsr cassette from the Nramp1-KO vector (Peracino et al., 2006). Clones were analyzed by PCR and positive ones further confirmed by Southern blot analysis.

Gene expression analysis by quantitative PCR

Total RNA was extracted with TRIzol, according to manufacturer instructions (Invitrogen, Carlsbad, CA, USA) from cells at different developmental times and analyzed by quantitative PCR. The RNA was treated with Dnase and retrotranscribed with random hexamer primers and Multiscribe Reverse transcriptase (High Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA), in accordance with manufacturer's instructions. Expression levels of *nramp1*, *nramp2* and reference *histone H3* were evaluated with SYBR technology with optimized PCR conditions and primer concentrations. Primer sequences were as follows: *nramp1* FW: CATCGGCTGCTGATTTCCTTAT, *nramp1* RW: ATAAACCGATTGCCCAA-ATGTATT; *nramp2* FW: CGAGGTTTCTCTGGTTTCG, *nramp2* RW: CCAACGATGCCAGTTGCA, *H3* FW: CGCACGTAAATCAACTGGTG, *H3* RW: GGAAACGAAGATCGGTTTTG. Melting curve analysis and efficiency evaluation were performed for all the amplicons. Quantitative PCR (qPCR) was carried-out on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) in 384-well plates assembled by Biorobot 8000 (Qiagen, Germantown, ML, USA). Baselines and thresholds for Ct calculation were set up manually with the ABI Prism SDS 2.1 software.

In vivo microscopy, fluorescence imaging and immunofluorescence

Living cells expressing Nramp1(C)- or Nramp2(C)-GFP were incubated with 1 mg/ml TRITC-dextran for 5 min under shaking, washed and plated on 6×6 cm glass coverslip equipped with a 5-cm diameter Plexiglas ring. For staining acidic vesicles, cells were incubated with 0.1 µM Neutral Red. Confocal series images were taken on an inverted Zeiss LSM510 microscope as described (Peracino et al., 2006). For immunofluorescence studies, cells were fixed with cold methanol, incubated with antibodies against the V-H⁺-ATPase subunit vatA and mounted with Gelvatol as described (Peracino et al., 1998). TRITC-labeled goat anti-mouse

(Jackson ImmunoResearch, West Grove, PA, USA) was used as secondary antibody.

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